Endogenous substrate proteins for Ca^{2+} -calmodulin-dependent, Ca^{2+} -phospholipid-dependent and cyclic AMP-dependent protein kinases in mouse pancreatic islets

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The occurrence of endogenous substrate proteins for Ca^{2+} -dependent protein kinase, augmented by either phospholipid or calmodulin, and for cyclic AMP-dependent protein kinase was examined in homogenates and subcellular fractions of mouse pancreatic islets. Islet protein phosphorylation was enhanced by Ca^{2+} -calmodulin; the major endogenous substrates in the homogenate were two proteins of M_r 53 000 and 100 000. The M_r -100 000 phosphoprotein was localized to a 27 000g-supernatant fraction, whereas the M_r -53 000 phosphoprotein was present in a 27 000g particulate fraction of mouse islets. In the presence of Ca^{2+} , phosphatidylserine stimulated phosphorylation of 15 proteins, of M_r 17 000–190 000, in a 27 000g-supernatant fraction. No effects of Ca^{2+} plus phosphatidylserine were observed in a 27 000g particulate fraction of mouse islets. Examination of cyclic AMP-dependent protein phosphorylation revealed five substrate proteins, of M_r 23 000–72 000, present in the 27 000g supernatant of mouse islets. No common substrates for either the two Ca^{2+} -dependent phosphorylation systems or for the cyclic AMP-dependent and the Ca^{2+} -calmodulin-dependent phosphorylation systems were noted. On the other hand, the actions of the cyclic AMP-sensitive and the Ca^{2+} -phospholipid-sensitive systems may be overlapping, since two common substrates for them were noted in the 27 000g-supernatant fraction. The results are consistent with the hypothesis that protein phosphorylation may play a role in the regulation of insulin secretion by Ca^{2+} and cyclic AMP. The extensive stimulatory effect of phosphatidylserine furthermore suggests that the Ca^{2+} -phospholipid-sensitive protein kinase may prove to be a prominent phosphorylation system in pancreatic islets.

An increase in the cytosolic concentration of Ca^{2+} is believed to trigger insulin release in the pancreatic β -cell (Hedekov, 1980). Details of the mechanism that produces this elevation of cytosolic Ca^{2+} in response to physiological stimuli, and the means by which this second messenger initiates secretion, remain unknown. One phenomenon that seems to be important in the Ca^{2+} -mediated regulation of secretion by cells is the phosphorylation of endogenous substrate proteins by Ca^{2+} -dependent protein kinases. Thus Ca^{2+} -dependent protein phosphorylation has been shown to accompany histamine secretion from mast cells

(Sieghart *et al.*, 1978), neurotransmitter release from synaptic vesicles (DeLorenzo & Freedman, 1978) and 5-hydroxytryptamine release from platelets (Kaibuchi *et al.*, 1982). Similarly insulin release from hamster insulinoma cells (Schubart *et al.*, 1980b) and from rat islets (Colca *et al.*, 1983) has been shown to coincide with Ca^{2+} -dependent phosphorylation of endogenous proteins.

One mediator of the regulatory effects of Ca^{2+} on protein phosphorylation and insulin secretion may be calmodulin. Several calmodulin-sensitive protein kinase systems have been defined. These include phosphorylase kinase (Cohen *et al.*, 1978), myosin light-chain kinase (Dabrowska *et al.*, 1978), tubulin kinase (Burke & DeLorenzo, 1982) and phosphorylation of endogenous proteins in several tissues (Schulman & Greengard, 1978;

Abbreviations used: Tes, 2-[[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino]ethanesulphonic acid; SDS, sodium dodecyl sulphate.

Yamauchi & Fujisawa, 1979; DeLorenzo *et al.*, 1979; Landt & McDonald, 1980) including rat islets (Harrison & Ashcroft, 1982) and insulinoma cells (Schubart *et al.*, 1980a; Brocklehurst & Hutton, 1983).

A distinct type of Ca^{2+} -dependent protein kinase which utilizes phospholipid rather than calmodulin as cofactor has also been described (Takai *et al.*, 1979a). This enzyme has an absolute requirement for Ca^{2+} and phospholipid, particularly phosphatidylserine (Takai *et al.*, 1979b; Kaibuchi *et al.*, 1981). Several reports furthermore suggest that unsaturated diacylglycerol, transiently formed during the receptor-mediated turnover of phosphatidylinositol, increases the affinity of this enzyme for Ca^{2+} as well as for phospholipid, and thus selectively initiates activation of this protein kinase (Takai *et al.*, 1979b; Kishimoto *et al.*, 1980). Some evidence suggests that the enzyme activated in this way may play a role in 5-hydroxytryptamine release from platelets (Sano *et al.*, 1983).

The major aim of the present study was to examine Ca^{2+} -phospholipid-stimulated endogenous protein phosphorylation in mouse islets. The results show the presence of phospholipid-sensitive Ca^{2+} -dependent protein kinase in mouse islets, in accordance with results previously reported for rat islets (Tanigawa *et al.*, 1982), and demonstrate the presence of a wide variety of endogenous substrate proteins for this protein kinase which are different from the substrate proteins for both the Ca^{2+} -calmodulin-sensitive and the cyclic AMP-sensitive protein kinase systems in pancreatic islets.

Experimental

Materials

Crude bacterial collagenase and cyclic AMP were obtained from Boehringer, Mannheim, Germany. Tes, EGTA, benzamidine, dithiothreitol, phospholipids, diolein, polymyxin B sulphate, lysine-rich histone (III-S), and ATP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 3-Isobutyl-1-methylxanthine was from EGA-Chemie, Steinheim/Albuch, Germany. Tri-fluoperazine was given by Rhône-Poulenc, Pharma Norden A/S, Copenhagen, Denmark. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 3000 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Calmodulin was prepared from bovine pancreas (Vandermeers *et al.*, 1977). All other chemicals were of analytical grade, obtained from ordinary commercial sources.

Preparation of islets

Islets were prepared by using collagenase digestion (Capito & Hedekov, 1977) of the pancreases

of male albino mice (approx. 26 g body wt.) fed *ad libitum* on a standard laboratory diet.

Preparation of islet particulate and soluble fractions

A group of 500 mouse islets was collected in 100 μl of 25 mM-Tes buffer, pH 6.90, containing 5 mM- MgCl_2 , 1 mM-EGTA, 0.1 mM-dithiothreitol and 10 mM-benzamidine. A homogenate was prepared by sonication (10 s, 40 W). For the preparation of subcellular fractions, the homogenate was centrifuged for 20 min at 27000 g at 4°C. The supernatant was retained for later use, and the pellet was resuspended and centrifuged once more in 100 μl of the same Tes buffer. After the last centrifugation and resuspension in 50 μl of the above Tes buffer, the particulate fraction and the supernatant from the first centrifugation of the homogenate (cytosol) were frozen in liquid N_2 and stored at -80°C until the next day. Protein was measured with bovine serum albumin as standard, as described by Lowry *et al.* (1951).

Phosphorylation assay

Samples of islet homogenate (corresponding to 50 islets), cytosol (corresponding to 50 islets) or particulate fraction (corresponding to 100 islets) were preincubated for 2 min in a reaction mixture (final volume 50 μl) containing 25 mM-Tes buffer, pH 6.90, 5 mM- MgCl_2 , 1 mM-EGTA, 0.1 mM-dithiothreitol and 10 mM-benzamidine, and further additions as indicated in the text and Figures. The reaction was started by addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (4–12 Ci/mmol, final concn. 10 μM). The reaction was carried out at 30°C for 2 min and terminated by addition of 50 μl of a solution containing 40 mM-Tris/HCl, pH 7.80, 4% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.016% (w/v) Bromophenol Blue, and heating at 95°C for 3 min. After cooling, the samples were left at room temperature overnight before electrophoresis on SDS/polyacrylamide gels.

To determine the subcellular distribution of the Ca^{2+} -phospholipid-dependent protein kinase, 0.2 mg of lysine-rich histone/ml was included in the reaction mixture described above. The particulate fraction and the homogenate were incubated with 0.3% (v/v) Triton X-100 for 1 h at 4°C before assay. Phosphorylation was performed at 30°C for 2 min with or without 1.1 mM- CaCl_2 and with or without 5 μg of phosphatidylserine. The reaction was stopped by addition of 5% (w/v) trichloroacetic acid containing 0.25% (w/v) sodium tungstate, with bovine serum albumin (0.3 mg) as a carrier protein, and the acid-precipitable radioactivity was determined, as described by Kuo *et al.* (1980). The enzyme activity, expressed as the Ca^{2+} -stimulated activity in the presence of phosphatidylserine, which was corrected for the Ca^{2+} -

stimulated activity in the absence of phosphatidylserine, was linear as a function of the incubation time and the amount of islet protein.

SDS/polyacrylamide-gel electrophoresis and autoradiography

Samples (75 μ l) were electrophoresed in SDS/polyacrylamide slab gels by a modification of the method of Laemmli (1970). The separation gel contained 7.5% (w/v) acrylamide, 0.375M-Tris/HCl, pH8.7, and 0.1% (w/v) SDS. The stacking gel contained 5% acrylamide, 0.127M-Tris/HCl, pH7.8, and 0.1% SDS. The electrophoresis buffer was 0.025M-Tris/0.192M-glycine buffer, pH8.3, containing 0.1% SDS. The electrophoresis was run at a constant current of 7.5mA and 20mA for the passage of proteins through the stacking and separating gel respectively. After staining with Coomassie Brilliant Blue, the gels were dried and then subjected to autoradiography with Kodak NS-5T X-ray film.

M_r values of the phosphoproteins were estimated by using the following proteins, with the indicated M_r values, as standards: myosin, 200000; β -galactosidase, 116250; phosphorylase *b*, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soya-bean trypsin inhibitor, 21500; and lysozyme, 14400.

The autoradiograms shown are representative of at least three similar experiments.

Results

Ca^{2+} -dependent phosphorylation in cytosol

Incubation of mouse islet cytosol (27000g supernatant of sonicated mouse islets) with [γ - 32 P]ATP resulted in incorporation of label into endogenous proteins (Fig. 1). The presence of Ca^{2+} markedly stimulated incorporation of 32 P into two protein bands, with estimated M_r values of 75000 and 100000. Addition of exogenous calmodulin did not further enhance the phosphorylation observed in the presence of Ca^{2+} alone, but rather decreased the incorporation of 32 P into the M_r -75000 protein band (Fig. 1). Trifluoperazine (100 μ M) abolished the Ca^{2+} -stimulated phosphorylation of both the M_r -75000 and the M_r -100000 proteins (results not shown).

Phosphorylation and autoradiography of the proteins in the cytosol fraction revealed ten proteins, with M_r values of 38000, 43000, 48000, 66000, 75000, 120000, 155000, 170000, and two of 190000, which were phosphorylated on addition of Ca^{2+} plus phosphatidylserine (Fig. 1). Dephosphorylation of one protein with an estimated M_r of 44000 was also observed with Ca^{2+} plus phosphatidylserine (Fig. 1). Phosphatidylserine alone stimulated the incorporation of 32 P into the M_r -

Fig. 1. Autoradiogram showing effects of Ca^{2+} , phosphatidylserine and calmodulin on endogenous protein phosphorylation in mouse islet cytosol

Preparation of cytosol, incubation conditions for phosphorylation, and subsequent SDS/polyacrylamide-gel electrophoresis and autoradiography of the phosphorylated samples were as described in the Experimental section. Additions were made as indicated and consisted of $CaCl_2$ (1.1mM, corresponding to 100 μ M ionized Ca^{2+}), phosphatidylserine (PS, 5 μ g), and calmodulin (CDR, 1 μ g). Phosphatidylserine was suspended in 25mM-Tes buffer, pH6.9, by sonication (1min, 40W) at 0°C before use in the assay. EGTA (1mM) was included in all cases. The incubation was performed for 2min at 30°C. The amount of protein applied to each well was 20 μ g. An example of the protein staining is shown to the right of the autoradiogram.

48000, -66000, -75000 and -155000 proteins. However, maximum effect on all substrates was only observed on addition of both Ca^{2+} and phosphatidylserine (Fig. 1).

Several smaller proteins, which migrated along with the tracking dye at the front of the 7.5% gel and were specific to stimulation by Ca^{2+} plus phosphatidylserine (Fig. 1), were determined to have M_r 17000, 19000, 20000, 28000 and 30000 in separate experiments with 16% gels (Fig. 2). The 32 P label associated with the M_r -17000 protein was not due to phosphorylation of calmodulin (estimated M_r 18000), since addition of calmodulin in combination with Ca^{2+} plus phosphatidylserine did not further increase the 32 P label at this position (Fig. 2).

Phosphorylation in the presence of 5 μ g of phosphatidylserine and 1.1mM- $CaCl_2$ at 1mM-EGTA for 2min (Figs. 1 and 2) were the optimum conditions for demonstration of Ca^{2+} -phospho-

Fig. 2. Effects of Ca^{2+} , phosphatidylserine and calmodulin on endogenous protein phosphorylation in mouse islet cytosol

The experimental conditions and abbreviations were the same as in Fig. 1, except that the separating gel was 16% acrylamide. An example of the protein staining is shown to the right of the autoradiogram.

lipid-sensitive protein phosphorylation in the cytosol. Maximum phosphorylation of the M_r -190000 proteins was obtained after 1 min of incubation, and tended to decrease after 10 min of incubation. The Ca^{2+} -dependent phosphorylations were all sensitive to free Ca^{2+} in the micromolar range. Addition of 1 mM- CaCl_2 at 1 mM-EGTA, which corresponds to an estimated free Ca^{2+} concentration of 20 μM (Flodgaard & Fleron, 1974), was able to elicit maximum phosphorylation of all substrates for the phospholipid-dependent phosphorylation system. High Ca^{2+} concentrations, e.g. 2 mM- CaCl_2 , slightly decreased the incorporation of label into all substrates compared with 1.1 mM- CaCl_2 .

Ca^{2+} -phospholipid-stimulated protein phosphorylation, identical with that induced by phosphatidylserine, was also induced by phosphatidylinositol (5 μg), whereas phosphatidylcholine (5 μg) and phosphatidylethanolamine (5 μg) were without effects. Furthermore, separate experiments showed that diolein (2 μg) in the presence of Ca^{2+} (1.1 mM- CaCl_2) gave a stimulated protein-phosphorylation pattern, which was identical with the Ca^{2+} -phospholipid-stimulated protein phosphorylation in the cytosol. However, diolein did not further potentiate the Ca^{2+} -phospholipid-dependent phosphorylation when added in combination with both Ca^{2+} (1.1 mM- CaCl_2) and phosphatidylserine (5 μg). Both trifluoperazine (250 μM) and polymyxin B (100 μM) abolished the phosphorylations observed in the presence of 1.1 mM- CaCl_2

Fig. 3. Effects of Ca^{2+} , phosphatidylserine, and calmodulin on endogenous protein phosphorylation in mouse islet homogenate

Homogenate was prepared as described in the Experimental section. The incubation conditions and the components added to the incubation as indicated were the same as described in Fig. 1. The amount of protein applied to each well was 35 μg . An example of the protein staining is shown to the right of the autoradiogram.

and phosphatidylserine (5 μg). This specificity towards phospholipids and inhibition with trifluoperazine and polymyxin B agree with the well-documented characteristics for Ca^{2+} -phospholipid stimulation of the phospholipid-dependent protein kinase from other tissues (Takai *et al.*, 1979b; Mori *et al.*, 1980; Mazzei *et al.*, 1982). Thus the observed phosphorylation pattern most probably reflects Ca^{2+} -phospholipid-stimulated protein kinase activity in the cytosol.

Ca²⁺-dependent phosphorylation in whole homogenate and particulate fraction

Phosphorylation experiments on whole homogenates of mouse islets did not reveal further Ca^{2+} -phospholipid-dependent protein phosphorylation when compared with the cytosolic fraction of mouse islets. Indeed several of the substrates for the Ca^{2+} -phospholipid-dependent protein kinase that were demonstrated in the cytosol could not be detected in the whole homogenate (Fig. 3). However, increased labelling of the M_r -66000, -120000, -155000 and -190000 proteins and decreased labelling of the M_r -44000 protein were also demonstrable in the whole homogenate on addition of Ca^{2+} plus phosphatidylserine. This apparent decline in substrates for the Ca^{2+} -phospholipid-dependent protein kinase, which

may be due to the combined action of ATP depletion and of active protein phosphatases in the homogenate, was not prevented by incubation for shorter periods ($\frac{1}{2}$ and 1 min) or by inclusion of the phosphatase inhibitor F^- (40 mM) in the incubation medium.

Addition of exogenous calmodulin to the whole homogenate revealed an additional substrate for the Ca^{2+} -calmodulin-sensitive protein kinase in comparison with the cytosol (Fig. 3). This protein, which has an estimated M_r of 53000, was specifically phosphorylated in the presence of calmodulin, since neither Ca^{2+} nor Ca^{2+} plus phosphatidylserine increased the incorporation of ^{32}P into this region.

Phosphorylation and autoradiography of the proteins in the 27000g particulate fraction of mouse islets showed that the M_r -53000 protein as well as its protein kinase were located in this fraction (Fig. 4). No substrate proteins for the phospholipid-sensitive system were observed in the particulate fraction, neither after 2 min of incubation (Fig. 4) nor after $\frac{1}{2}$ or 1 min of incubation (results not shown). Diolein alone or in combination with phosphatidylserine was not able to elicit Ca^{2+} -phospholipid-dependent protein phosphorylation in the particulate fraction. The particulate fraction contained $29 \pm 2\%$ (mean $\pm$ S.E.M., $n = 3$) of the phospholipid-dependent

protein kinase activity of mouse islets. Furthermore, solubilization of the particulate fraction with Triton X-100 before phosphorylation did not reveal any endogenous substrate proteins for the phospholipid-sensitive enzyme when assayed in the absence or presence of F^- (40 mM). Thus this lack of protein phosphorylation suggests that there are no substrate proteins for the phospholipid-dependent enzyme in the particulate fraction of mouse islets.

Cyclic AMP-dependent phosphorylation in whole homogenate and subcellular fractions

Cyclic AMP has been shown to have a marked potentiating effect on the ability of glucose and several other agents to stimulate insulin release from islets (Hedekov, 1980). The mechanism by which cyclic AMP potentiates insulin secretion is not understood, but there is ample evidence that the action of cyclic AMP in eukaryotic cells is mediated by activation of cyclic AMP-dependent protein kinases (Greengard, 1978).

To investigate the relationship of Ca^{2+} -dependent protein phosphorylation with cyclic AMP-dependent protein phosphorylation in mouse is-

Fig. 4. Effects of Ca^{2+} , phosphatidylserine and calmodulin on endogenous protein phosphorylation in mouse islet particulate fraction

The particulate fraction was prepared as described in the Experimental section. The incubation conditions and the components added to the incubation as indicated were the same as described in Fig. 1. The amount of protein applied to each well was 20 μ g. An example of the protein staining is shown to the right of the autoradiogram.

Fig. 5. Effect of cyclic AMP on endogenous protein phosphorylation in mouse islet cytosol

Preparation of cytosol, incubation conditions for phosphorylation, and subsequent SDS/polyacrylamide-gel electrophoresis and autoradiography of the phosphorylated samples were as described in the Experimental section. Additions were made as indicated, and consisted of 3-isobutyl-1-methylxanthine (IBMX; 1 mM) and cyclic AMP (5 μ M). The amount of protein applied to each well was 20 μ g. An example of the protein staining is shown to the right of the autoradiogram.

lets, experiments were performed in the absence of Ca^{2+} to examine cyclic AMP-dependent protein kinase activity. Addition of cyclic AMP ($5\ \mu\text{M}$) in the presence of 3-isobutyl-1-methylxanthine (1 mM), included to inhibit cyclic AMP phosphodiesterase activity, resulted in incorporation of label into endogenous proteins. The main effects of cyclic AMP in a whole homogenate of mouse islets (results not shown) and in the cytosolic fraction of mouse islets (Fig. 5) were the phosphorylation of three proteins, with estimated M_r values of 50 000, 60 000 and 72 000. Furthermore, separate experiments using 16% gels revealed two smaller proteins in the cytosol, with M_r values of 23 000 and 28 000, which were phosphorylated in the presence of cyclic AMP. Of these, only the M_r -28 000 and -72 000 proteins may be substrates for the Ca^{2+} -dependent phosphorylation systems, since Ca^{2+} -phosphatidylserine stimulated incorporation of ^{32}P into proteins with similar M_r values.

No effects of cyclic AMP on phosphorylation of proteins in the particulate fraction of mouse islets were noted.

Discussion

The present findings demonstrate that Ca^{2+} -dependent phosphorylation of endogenous proteins in pancreatic islets is regulated independently by phospholipids and calmodulin. None of the substrates for the Ca^{2+} -phospholipid-sensitive system were phosphorylated by the Ca^{2+} -calmodulin-sensitive system. Ca^{2+} alone stimulated the incorporation of ^{32}P into two proteins with M_r values of 75 000 and 100 000 in the cytosol (Fig. 1). The phosphorylation of the M_r -75 000 protein by Ca^{2+} was most probably mediated by endogenous phospholipids in the cytosol, since the phosphorylation of this protein was further enhanced by phosphatidylserine and slightly inhibited by calmodulin (Fig. 1). On the contrary, phosphatidylserine inhibited the Ca^{2+} -stimulated incorporation of ^{32}P into the M_r 100 000 protein, which seemed to be mediated by endogenous calmodulin (Fig. 1).

The present findings for calmodulin agree with previous results obtained in a whole homogenate of rat islets (Harrison & Ashcroft, 1982). This agreement includes the observed phosphorylation of both the M_r -53 000 and the M_r -100 000 proteins. Furthermore, the M_r -100 000 protein present in the cytosolic fraction may be identical with the M_r -98 000 protein previously demonstrated to be phosphorylated in the presence of Ca^{2+} -calmodulin in cytosol from hamster insulinoma cells (Schubart *et al.*, 1980a).

The results confirm that pancreatic islets contain endogenous substrates for cyclic AMP-dependent protein kinase, which may play a role in the

stimulation of insulin release by cyclic AMP (Harrison & Ashcroft, 1982). The observation that cyclic AMP stimulates phosphorylation of proteins distinct from the substrate proteins for both the phospholipid-stimulated and the calmodulin-stimulated Ca^{2+} -dependent phosphorylation systems suggest independent roles for either system in pancreatic islets. The actions of the cyclic AMP-dependent and the Ca^{2+} -phospholipid-dependent system, on the other hand, may be overlapping, since common substrate proteins for these two systems were also noted.

The observation by Harrison & Ashcroft (1982) of a common M_r -53 000 substrate protein for the cyclic AMP-sensitive and the Ca^{2+} -calmodulin-sensitive phosphorylation systems in rat islets was not supported by the present findings in mouse islets. In our hands, the main effects of cyclic AMP were the phosphorylation of two cytosolic proteins surrounding the M_r -53 000 protein, i.e. one of M_r 50 000 and one of M_r 60 000. Most probably the Ca^{2+} -calmodulin-regulated M_r -53 000 protein, which could be localized to the particulate fraction of mouse islets (Fig. 4), may be identical with the Ca^{2+} -calmodulin-regulated M_r -54 000 protein present in the microsomal fraction of rat islets, which is insensitive to cyclic AMP (Colca *et al.*, 1983).

Unlike the Ca^{2+} -calmodulin-sensitive system, which was present in both particulate and cytosolic fractions, the substrates for the phospholipid-sensitive system seem to be mainly cytosolic in origin. The extensive number of substrates for the Ca^{2+} -phospholipid-stimulated protein kinase activity in pancreatic islets suggest that this protein kinase may prove to be a prominent phosphorylation system in pancreatic islets. Furthermore, the activation of this protein kinase may be coupled to glucose-induced phosphatidylinositol turnover previously demonstrated in pancreatic islets (Clements *et al.*, 1981), since diolein markedly stimulated endogenous protein phosphorylation in the cytosol. Regarding the diolein-stimulated phosphorylation, it should be noted, however, that this phosphorylation was demonstrated in the absence of added phospholipids and that it was not investigated whether this effect might be explained by a diacylglycerol kinase-catalysed formation of phosphatidic acid which in the presence of Ca^{2+} may activate Ca^{2+} -phospholipid-dependent protein kinase activity (Takai *et al.*, 1979a).

The present studies have clearly demonstrated the occurrence in pancreatic islets of a Ca^{2+} -dependent phosphorylation system for endogenous proteins stimulated specifically by phospholipid, which was distinguishable from the Ca^{2+} -calmodulin-stimulated and the cyclic AMP-stimulated systems. At this point the functional signifi-

cance of and the interrelationship between the three putative phosphorylation systems are not clear. Clarification of these problems may be facilitated by exploring phosphorylation of specific endogenous proteins in response to physiological stimuli and pharmacological agents in intact-cell preparations.

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